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A Genetically Engineered Waterfowl Influenza Virus with a Deletion in the Stalk of the Neuraminidase Has Increased Virulence for Chickens[▽]

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A deletion of about 20 amino acids in the stalk of the neuraminidase (NA) is frequently detected upon transmission of influenza A viruses from waterfowl to domestic poultry. Using reverse genetics, a recombinant virus derived from a wild duck influenza virus isolate, A/Mallard/Marquenterre/Z237/83 (MZ), and an NA stalk deletion variant (MZ-delNA) were produced. Compared to the wild type, the MZ-delNA virus showed a moderate growth advantage on avian cultured cells. In 4-week-old chickens inoculated intratracheally with the MZ-delNA virus, viral replication in the lungs, liver, and kidneys was enhanced and interstitial pneumonia lesions were more severe than with the wild-type virus. The MZ-delNA-inoculated chickens showed significantly increased levels of mRNAs encoding interleukin-6 (IL-6), transforming growth factor-β4 (TGF-β4), and CCL5 in the lungs and a higher frequency of apoptotic cells in the liver than did their MZ-inoculated counterparts. Molecular mechanisms possibly underlying the growth advantage of the MZ-delNA virus were explored. The measured enzymatic activities toward a small substrate were similar for the wild-type and deleted NA, but the MZ-delNA virus eluted from chicken erythrocytes at reduced rates. Pseudoviral particles expressing the MZ hemagglutinin in combination with the MZ-NA or MZ-delNA protein were produced from avian cultured cells with similar efficiencies, suggesting that the deletion in the NA stalk does not enhance the release of progeny virions and probably affects an earlier step of the viral cycle. Overall, our data indicate that a shortened NA stalk is a strong determinant of adaptation and virulence of waterfowl influenza viruses in chickens.

In the waterfowl reservoir, influenza A viruses are enzootic and infections are usually asymptomatic. The viruses replicate preferentially in the intestinal tract and are transmitted by the fecal-oral route. Phylogenetic analyses of amino acid changes show that influenza viruses in wild aquatic birds have low evolution rates, suggesting that they are in evolutionary stasis. Upon transmission to domestic poultry, rapid evolution occurs (63, 79). The replication of influenza viruses of duck origin in chickens is generally limited to the respiratory and gastrointestinal tracts and causes mild or no symptoms. However, sustained replication of viruses of the H5 or H7 subtype may lead to the emergence of highly pathogenic influenza viruses, which cause devastating epizootics (for a review, see reference 5). Mutations acquired upon replication in domestic poultry might also increase the potential for adaptation of avian influenza viruses to other species, including humans (20, 21, 50), which raises public health concerns.

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The viral surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) are major determinants in the interspecies transmission and adaptation of influenza A viruses to a new host (for a review, see reference 46). The HA binds to the sialic acids (SA) linked to cellular membrane glycoproteins or glycolipids, whereas the sialidase activity of the NA facilitates the release and diffusion of progeny virions. SA are usually attached to a galactose moiety via an α2,3 or α2,6 glycosidic linkage. Avian viruses bind preferentially to SA-α2,3-galactose, whereas human viruses bind preferentially to SA-α2,6-galactose (13, 37, 56). This receptor binding specificity correlates with the relative predominance of SA-α2,3-galactose and SA-α2,6-galactose at the sites of viral multiplication in ducks and in humans, respectively (14, 28), and involves specific residues in the receptor binding site of the HA (37, 70). Viruses isolated from terrestrial poultry bind preferentially to SA-α2,3-galactose, but they differ from duck viruses by an enhanced binding to receptors in which the penultimate saccharide is sulfated and/or fucosylated (22, 23). Changes in the receptor binding site or at potential glycosylation sites of the HA that could modulate the binding of the HA to the receptors have been associated with the adaptation of duck viruses to poultry (3, 21, 36). A deletion of about 20 amino acids in the stalk region of the NA has also been frequently detected concomitant with the adaptation of duck viruses to poultry (3, 4, 25, 36, 62). In particular, it is a feature of the highly pathogenic H5N1 viruses

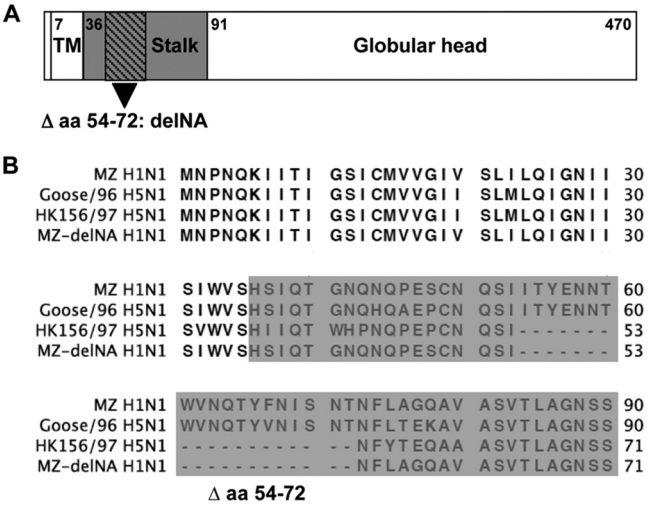

FIG. 1. Wild-type and deleted variants of the MZ-NA protein. (A) Schematic representation of the wild-type MZ-NA protein (470 amino acids [aa]). The domain corresponding to the stalk (amino acids 36 to 90) is represented in gray. The region of the stalk which is deleted in the MZ-delNA variant (amino acids 54 to 72) is represented by a hatched box. TM, transmembrane domain. (B) Amino acid sequence alignment of the NAs from MZ (H1N1), MZ-delNA (H1N1), A/Goose/Guandong/1/96 (H5N1), and A/Hong Kong/156/97 (H5N1) viruses. The domain corresponding to the stalk is highlighted in gray. The residues that are deleted in the NAs of MZ-delNA and A/Hong Kong/156/97 are represented by dashes.

that have become endemic in poultry in southern Asia since 2003 and have been causing sporadic human cases (26). The stalk is a flexible region which separates the globular, enzymatically active head of the tetrameric NA from the hydrophobic transmembrane domain (Fig. 1A). The biological significance of the selection of variants with a shorter NA stalk is still unclear. Shortening of the stalk was found to decrease the ability of the NA to release the virus from cells (2, 6, 17, 25, 36, 39), and it was suggested that such a decreased activity of the NA could counterbalance a reduced binding of the HA to sialic acids expressed in poultry (2, 40, 71). The NA stalk length was found to have little effect on the efficiency of replication of a highly pathogenic H5N1 virus in poultry (39). However, it is possible that the presence of a multibasic site on the H5, a strong determinant of virulence, might be masking the effect, if any, of a shortened NA stalk.

In the present study, we used a low-pathogenic duck influenza A(H1N1) virus isolate to investigate possible mechanisms involved in the selection of influenza viruses with a deletion in the stalk of the NA upon transmission from the waterfowl reservoir to chickens. Reverse genetics was used to produce a wild-type (wt) virus and a variant with a 19-amino-acid deletion in the stalk of the NA. The rationale for using an H1N1 virus was that a slight increase in viral fitness of the deleted variant, if any, would be more readily detected if the wild-type virus showed a low replication potential in chickens. *In vitro* and *in vivo* approaches were developed to examine the effect of the deletion in the stalk of the NA on the biological properties of the enzyme, on viral infectivity in avian cultured cells, and on viral infectivity and pathogenicity in chickens.

MATERIALS AND METHODS

Ethics statement. All animals were handled in strict accordance with good animal practice as defined by the relevant national and/or local animal welfare bodies, and all animal work was approved by the appropriate committee (Comité Régional d'Ethique pour l'Expérimentation Animale, Région Centre).

Viruses and cells. The H1N1 influenza A virus A/Mallard/Marquenterre/Z237/83 (MZ) was isolated by the National Influenza Center (Northern France) at the Institut Pasteur in Paris (France) and passaged twice on 11-day-old embryonated eggs.

293T, DF1, and HD11 cells and primary duck embryonic fibroblasts (DEF) (a gift from R. Volmer, Ecole Nationale Vétérinaire, Toulouse, France) were grown in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). QT6 cells were grown in Ham F-10 medium supplemented with 10% FCS, 1% chicken serum, and 2% tryptose phosphate broth. Primary chicken embryonic fibroblasts (CEF) were prepared using a standard procedure and grown in DMEM supplemented with 10% FCS and 1% chicken serum.

Plasmids. Expression plasmids pHMG-MZ-PB1, -PB2-627K, -PA, and -NP and reverse-genetics plasmids pPR7-MZ-PB1, -PB2, -PA, and -NP have been described earlier (33, 53). To generate the additional reverse-genetics plasmids pPR7-MZ-NA, -HA, -M, and -NS, cDNAs were prepared by reverse transcription of the viral RNA (vRNA), amplified using primers specific for the coding sequences (NA) or for the noncoding sequences (HA, M, and NS), subcloned into the pCI (NA) or pCR-2.1-TOPO plasmid (HA, M, and NS), and sequenced using a Big Dye terminator sequencing kit and an automated sequencer (Perkin-Elmer). GenBank accession numbers for the PB1, PB2, PA, NP, HA, NA, M, and NS segments of the MZ virus are DQ864506, DQ864507, DQ864508, DQ864509, GU066779 GU066780, GU066781, and GU066782, respectively. Plasmids showing the consensus coding sequences of MZ-NA, -HA, -M, and -NS were used as templates for a second amplification, using primers containing the 3′ and 5′ noncoding regions (NCR) of the corresponding genomic segments. The amplicons were cloned between two BbsI sites in the pPR7 plasmid. The pPR7-MZ-delNA plasmid was obtained by deletional mutagenesis of the pPR7-MZ-NA plasmid using the QuikChange site-directed mutagenesis kit (Stratagene) with the 5′-CCTGAATCATGCAATCAAAGCATAAATTTTCTTGCTGGGCAGGCTGTAGC-3′ primer and the reverse complementary primer. To generate the pCI-delNA plasmid, the coding sequence of delNA was amplified with appropriate primers and subcloned between the XhoI and NotI sites in the pCI plasmid. The Y406F mutation was introduced in the pCI-NA and pCI-delNA plasmids using the QuikChange site-directed mutagenesis kit (Stratagene). The sequences of the oligonucleotides used for amplification, mutagenesis, and sequencing can be provided upon request. The plasmid expressing MZ-HA was obtained by cloning a PCR fragment amplified from pPR7-MZ-HA between the BglII and NheI sites in the pVITRO2-blasti-mcs plasmid (InvivoGen). The pNL4-3-ΔENV-LucR plasmid drives the expression of a human immunodeficiency virus type 1 (HIV-1)-derived genome encoding *Renilla* luciferase instead of the HIV-1 *nef* gene and has been described earlier (47). All constructs were verified by sequencing as described above.

Generation of infectious recombinant viruses. The method used for the production of recombinant influenza viruses by reverse genetics was adapted from previously described procedures (18). Briefly, the four pHMG-PB1, -PB2-627K, -PA, and -NP plasmids together with the eight pPR7-MZ-PB1, -PB2, -PA, -NP, -NA or -delNA, -HA, -M, and -NS plasmids were cotransfected (0.5 μg each) into a subclonfluent monolayer of 293T cells cocultivated with CEF (4×10^5 293T cells with 6×10^5 CEF seeded in a 35-mm dish), using 10 μl of the FuGENE 6 transfection reagent (Roche). After 24 h of incubation at 37°C, the supernatant was removed, and cells were washed twice with DMEM and incubated for 48 h at 37°C in DMEM containing tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (Worthington) at a final concentration of 0.4 μg/ml. Supernatants were subsequently inoculated into 10-day-old embryonated chicken eggs. Allantoic fluids were tested individually for the presence of infectious virus by a standard hemagglutination assay using chicken red blood cells (CRBCs) (32). Viral titers in the positive samples were measured by plaque assay on QT6 cells as previously described (53). Working stocks of the MZ and MZ-delNA recombinant viruses were obtained after two additional rounds of amplification on 10-day-old embryonated chicken eggs at a multiplicity of infection (MOI) of 2 PFU/egg.

Viral infection of cultured cells. For viral growth assays, confluent monolayers of QT6, CEF, or DEF cells were infected at an MOI of 0.001 PFU/cell. Following 1 hour of adsorption at 37°C, cells were washed three times and then incubated with Ham F-10 (QT6) or DMEM (CEF and DEF) supplemented with 0.4 μg/ml of TPCK-trypsin (Worthington). Virus titers in the supernatants collected at

different time points were measured by plaque assays on QT6 cells as described previously (53). For cytokine induction assays, confluent monolayers of DF1 or HD11 cells were infected at an MOI of 3 PFU/cell. Following 1 hour of adsorption at 37°C, cells were washed twice and then incubated with complete culture medium for 6 h, when RNA analysis was performed.

NA activity assays. Subconfluent monolayers of 293T cells were transfected with pCI-NA plasmids by using the FuGENE 6 transfection reagent (Roche). At 48 h posttransfection, NA enzymatic activity in cell suspensions or cell extracts was measured, as described earlier (52). To prepare soluble NA-containing extracts, cells were harvested and resuspended in morpholineethanesulfonic acid (MES) buffer with 0.92 g/liter β-dodecyl-D-maltoside (MES-DM buffer). For enzymatic assays, cell suspensions or extracts were incubated with increasing concentrations (5 to 100 μM) of the fluorogenic substrate 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA) (Sigma), and the fluorescence of the released 4-methylumbelliferone was monitored using a Xenius spectrofluorometer (SAFAS). The kinetic parameters K_m and V_{max} were calculated by fitting the data to the appropriate Michaelis-Menten equations using KaleidaGraph software (Synergy Software). The steady-state levels of NA in MES-DM cell extracts were analyzed by Western blotting using a rabbit polyclonal antibody directed against the NA of A/Vietnam/1203/04 (a gift from N. Escriou, Institut Pasteur, Paris, France) or a monoclonal antiactin antibody (AC-15; Abcam) and Qdot-655-coupled secondary antibodies (Invitrogen). The fluorescent signals were quantified using a G-box (Syngene), and the NA signal was normalized to the actin signal.

To determine the rate of virus elution from chicken red blood cells (CRBCs), 50 μl of twofold serial dilutions of the viral stocks in phosphate-buffered saline (PBS)–0.9 mM $CaCl_2$, with or without 100 nM zanamivir (a gift from M. von Itzstein, Griffith University, Australia), was incubated with 50 μl of a 0.5% (vol/vol) suspension of CRBCs in U-bottom microtiter plates (36). Plates were left on ice for 1 h to allow virus adsorption to the CRBCs and then transferred to a water bath at 37°C. The decrease in HA titer, which reflected the NA-mediated virus elution from CRBCs, was monitored for 24 h.

Production of HIV-1-like particles expressing MZ-HA and NA. Subconfluent monolayers of DF1 cells seeded in six-well plates were cotransfected with 1.5 μg of pNL4-3-ΔENV-LucR and 1 μg of pVITRO2-blasti-mcs-MZ-HA in the absence or in the presence of 1 μg of the pCI vector encoding MZ-NA, MZ-delNA, MZ-NA-Y406F, or MZ-delNA-Y406F. The empty pCI vector was used as carrier plasmid DNA to normalize the quantity of DNA transfected per well. The Lipofectamine LTX transfection reagent (Invitrogen) was used according to the manufacturer's recommendations. After incubation at 37°C for 16 h, cells were washed with PBS, and fresh complete culture medium was added. In some wells, recombinant *Clostridium perfringens* neuraminidase (Worthington) or zanamivir was added at a final concentration of 60 mU/ml or 1 μM, respectively. Cell supernatants were recovered 24 h later and centrifuged for 10 min at 2,500 rpm to remove cell debris. The HIV-1 p24 antigen content in the supernatants, reflecting the amount of viral pseudoparticles (VPP) released, was measured by enzyme-linked immunosorbent assay (ELISA) (Innogenetics/Ingen).

Animal experiments. Four-week-old specific-pathogen-free (SPF) histocompatible B13/B13 White Leghorn chickens were housed in biosafety level 3 cabinets under negative pressure with HEPA-filtered air. Briefly, three groups of 15, 15, and 8 birds were inoculated intratracheally with 0.1 ml of the MZ or MZ-delNA virus stocks (10^7 50% egg infective doses [EID_{50}]/0.1 ml) or with 0.1 ml of virus-free allantoic fluid, respectively. Birds were monitored daily for clinical signs of disease. In each virus-inoculated group, three birds were euthanized at days 2 and 7 postinoculation (p.i.) and 5 birds at days 3 and 4 p.i. Two mock-inoculated birds were euthanized in parallel at days 2, 3, 4, and 7 p.i. For each tissue, including trachea, lung, liver, spleen, kidney, cecum, pancreas with duodenum, cecal tonsil, cloacal bursa, and brain, one sample was dry-frozen for virus isolation and viral RNA quantification, one was frozen with RNA-later solution (Ambion) for cytokine mRNA quantification, and one was frozen in nitrogen-cooled isopentane for immunohistochemistry, before being stored at −80°C until use. An additional sample of each tissue mentioned above was fixed in 10% neutral buffered formalin for histopathological evaluation. Tracheal and cloacal swabs were stored in virus transport medium (Viral-pack; Biomedics) at −80°C.

For virus isolation, the tissue samples were weighted, transferred in 1 ml of PBS, and dissociated mechanically using a tissue grinder (Retsch, Germany). Ten-day-old SPF embryonated chicken eggs were inoculated either with undiluted or 10-fold-diluted tissue homogenates or with swabs. At 4 days postinoculation, allantoic fluids were taken and assayed for the presence of virus by a standard hemagglutination test using CRBCs.

Histopathology and immunohistochemistry. Formalin-fixed tissues were embedded in paraffin wax, and 6 μm-thick sections were cut and routinely stained with hematoxylin-eosin-safran for histologic observation. Indirect immunofluorescence assays were performed on 8-μm-thick sections of frozen samples. Sections were pretreated with methanol at −20°C for 10 min and with 20% normal goat serum (Dako) at room temperature for 20 min. Sections were incubated with the IH13 or IG12 anti-NS1 (J. F. Vautherot and D. Marc, unpublished) or IA52 anti-NP (Argene) mouse monoclonal antibodies, diluted 1:1600, 1:400, and 1:200, respectively, in PBS–2% goat serum–2% bovine serum albumin (BSA) (Sigma) overnight at 4°C and with an Alexa 488-coupled secondary antibody (Invitrogen) diluted 1:300 in PBS–2% goat serum–2% BSA for 1 hour at room temperature. Nuclei were stained with 1 μM TO-PRO-3 (Invitrogen). Sections were mounted using Mowiol medium (Calbiochem) following the manufacturer's instructions and serial scanned using the He-Ne and argon ion lasers of a C1 Nikon laser scanning confocal microscope. An *in situ* cell death detection kit (Roche) was used on liver sections, following the manufacturer's instructions. The numbers of total and apoptotic nuclei per field were determined (coefficient of variation of 3.4%) after random selection of two microscopic fields. More than 400 nuclei were observed for each sample (490 ± 59 per sample).

Quantification of viral RNAs. The QiaAmp viral RNA mini kit (Qiagen) was used to prepare vRNAs from 140 μl of allantoic fluids or tissue homogenates, according to the manufacturer's recommendations. For sequence determination, the vRNAs prepared from allantoic fluids were reverse transcribed and amplified using the Titan one-step reverse transcription-PCR (RT-PCR) kit (Roche). Amplification products were purified using a QIAquick gel extraction kit (Qiagen) and sequenced as described above. The sequences of the oligonucleotides used for amplification and sequencing can be provided upon request.

For the quantification of M-vRNAs by real-time RT-PCR, the Superscript III Platinum one-step quantitative RT-PCR (qRT-PCR) (Invitrogen) and LightCycler480 instrument (Roche) were used. The sequences of the primers and probe used for detection were adapted from those described by Fouchier et al. (19) and can be provided upon request. A standard curve was obtained by subjecting 10^2 to 10^6 copies of an *in vitro*-transcribed M-vRNA to qRT-PCR in parallel. The number of M-vRNA copies/mg of tissue was calculated based on the tissue weight equivalent used for vRNA extraction and the fraction of vRNAs subjected to qRT-PCR. The positive cutoff was set at crossing-point values of ≤34, which corresponded to $\geq 2 \times 10^2$ copies of M-vRNA according to the standard curve and to 6.8×10^2 copies/mg in the lung samples and 3.4×10^2 copies/mg in the liver or kidney samples.

Quantification of cellular cytokine mRNAs. Total RNA was extracted from cells and tissues with the Tri Reagent solution, according to the manufacturer's recommendations (Sigma), and treated with RNase-free DNase I (Invitrogen). Reverse transcription was performed on 1 μg of RNA using the Superscript first-strand synthesis system for RT-PCR (Invitrogen) according to the manufacturer's recommendations. Amplification of the cDNA by qPCR (Chromo 4; Bio-Rad) was performed in triplicate, using 2 μl of the 10-fold-diluted cDNA sample (i.e., 1% of the reverse transcription products), 7.5 μl of 2× iQ Supermix SYBR green (Bio-Rad), 4 μl of ultrapure water (Invitrogen), and 0.75 μl of each oligonucleotide (10 μM) of pairs specific for the chicken interleukin-6 (IL-6), transforming growth factor-β4 (TGF-β4), alpha interferon (IFN-α), IFN-β, IFN-γ, IL-8, CCL5, CCL4, CCL20, and ubiquitin cDNAs. The sequences of the oligonucleotides can be provided upon request. For each target sequence, a standard curve was obtained by subjecting 1 to 10^6 copies of a corresponding DNA plasmid to qPCR in parallel. Only PCR products showing a unique temperature of fusion after raising the temperature to 95°C at a transition rate of 0.5°C/s were retained for further quantitative automated analysis with the Opticon Monitor 3 software (Bio-Rad). The concentrations of target cDNAs determined experimentally were normalized to 10^7 copies of ubiquitin cDNA as measured in the same sample.

Statistical analyses. Viral RNA copy numbers as determined by qRT-PCR on RNA extracted from the lungs, livers, and kidneys of inoculated animals were analyzed as follows: (i) values below the detection threshold, i.e., 200 copies, were arbitrarily replaced by 100, and (ii) copy numbers were converted to their $\log_{10}$ values, which were then expressed as a percentage of the maximum value observed in virus-inoculated birds for a given tissue on a given date (day 2, 3, or 4 p.i.). For each tissue, the two sets (MZ versus MZ-delNA) of grouped normalized values (day 2 plus 3 plus 4 p.i.) were compared using a Mann-Whitney test with a two-tailed *P* value.

Cytokine-mRNA copy numbers determined on lung samples from mock-, MZ-, or MZ-delNA-inoculated animals or on control, MZ-, or MZ-delNA-infected cells were first compared using a Kruskall-Wallis test in order to detect any virus-induced changes. The values obtained for the MZ- and MZ-delNA-inoculated animals or infected cells were further compared using a Mann-Whitney test with a two-tailed *P* value. Fold changes below a threshold value of 3 were considered nonsignificant.

Statistical analyses were carried out using the GraphPad Prism 5.0 software.

TABLE 1. Characterization of MZ and MZ-delNA viral stocks

Virus	EID_{50}/ml[a]	PFU/ml[b]	HA U[c]	M-vRNA copies/ml[d]
MZ	$10^{8.3}$	$10^{6.88}$	128	$10^{10.35}$
MZ-delNA	10^{9}	$10^{6.95}$	64	$10^{10.28}$

[a] EID_{50} titers were calculated using the Reed-Muench method after inoculation of serial dilutions of viral stocks into 10-day-old embryonated chicken eggs.
[b] PFU titers were determined by plaque assay on QT6 cells.
[c] HA titers were determined using a 0.5% suspension of chicken red blood cells.
[d] M-vRNA copies were determined by real-time quantitative RT-PCR following viral RNA extraction.

RESULTS

Production of the MZ and MZ-delNA recombinant viruses. The NA protein encoded by the A/Mallard/Marquenterre/Z237/83 (MZ) (H1N1) virus shows 95.5% homology with the NA protein of the A/Goose/Guandong/1/96 (H5N1) virus, a precursor of the highly pathogenic H5N1 viruses responsible for epizootics and human cases in Hong Kong in 1997 (77). Both viruses show an NA stalk that is about 55 amino acids in length (Fig. 1A and B). In comparison, the Hong Kong 1997 H5N1 viruses (e.g., the A/Hong Kong/156/97 isolate) as well as most highly pathogenic H5N1 viruses isolated from both poultry and humans since 2003 show a 19-amino-acid deletion in the NA stalk (Fig. 1B). This deletion of amino acids 54 to 72 (delNA) was introduced into the cDNA corresponding to the MZ-NA genomic segment (Fig. 1A and B). Reverse genetics was used to produce recombinant wild-type MZ and MZ-delNA viruses.

Cocultures of 293T cells and primary chicken embryonic fibroblasts (CEF) were transfected with a mixture of eight MZ-derived PolI plasmids, including the NA or delNA PolI plasmid, and four expression plasmids for the MZ-PB1, -PB2-627K, -PA, and -NP proteins, as described in Materials and Methods. The mutant MZ-PB2-627K protein was expressed instead of the wild-type MZ-PB2-627E protein, because it was expected to increase the activity of the reconstituted MZ polymerase in 293T cells (33) and thus to increase the efficiency of viral rescue. Still, the efficiency of reverse genetics was very low. It was likely limited by the low replicative potential of the recombinant MZ viruses, which encoded a wild-type PB2-627E protein, on 293T cells. No infectious virus could be detected in the reverse genetics transfection supernatants, and following inoculation of these supernatants into embryonated eggs, only 1 out of 10 allantoic fluids tested individually using a hemagglutination assay was positive for the presence of virus. This experimental procedure was thus considered to be equivalent to a limiting-dilution cloning. The viral stocks obtained upon amplification in embryonated chicken eggs were titrated using various assays (EID_{50} determination, plaque formation on QT6 cells, hemagglutination, and quantification of the M genomic segment by qRT-PCR) and found to be comparable for both viruses (Table 1). High infectious titers in the range of 10^8 to 10^9 EID_{50}/ml were obtained for both viruses.

The eight genomic segments of the recombinant viruses were extensively sequenced. A silent mutation that had been purposedly conserved on the PB2 reverse genetics plasmid in order to distinguish recombinant viruses from the parental virus (PB2 segment nucleotide A165G, PB2 residue Arg46), was present on the PB2 segments of both recombinant viruses. The sequences of the wild-type and delNA viruses showed no major differences, except for the deletion in the NA stalk. Notably however, sequence heterogeneity was detected at nucleotides 979 (C/T, corresponding to Thr/Ile at residue 316) and 1197 (A/T, corresponding to Ile/Phe at residue 389) of the HA gene in the MZ viral stock and at nucleotide 401 (A/C, corresponding to Ser/Arg at residue 123) in the HA gene of the MZ-delNA viral stock.

Growth properties of MZ and MZ-delNA on cultured avian cells. On the QT6 quail fibroblastic cell line, the recombinant MZ virus formed plaques of heterogeneous size with a mean diameter of 14.4 ± 6.7 mm, similar to that observed for the parental MZ virus (Fig. 2A). The recombinant MZ-delNA virus formed plaques significantly larger than the wild type, with a mean diameter of 25.2 ± 8.7 mm ($P < 0.001$) (Fig. 2A).

The growth kinetics of the recombinant MZ and MZ-delNA viruses were examined upon infection of QT6 cells, primary CEF or duck embryonic fibroblasts (DEF). Cell monolayers were infected at a low multiplicity of infection (0.001 PFU/cell) and then incubated at 37°C. Supernatants were collected at 12, 24, 36, and 48 h p.i., and viral titers were determined by plaque assays on QT6 cells. On CEF and QT6 cells, the viral titers

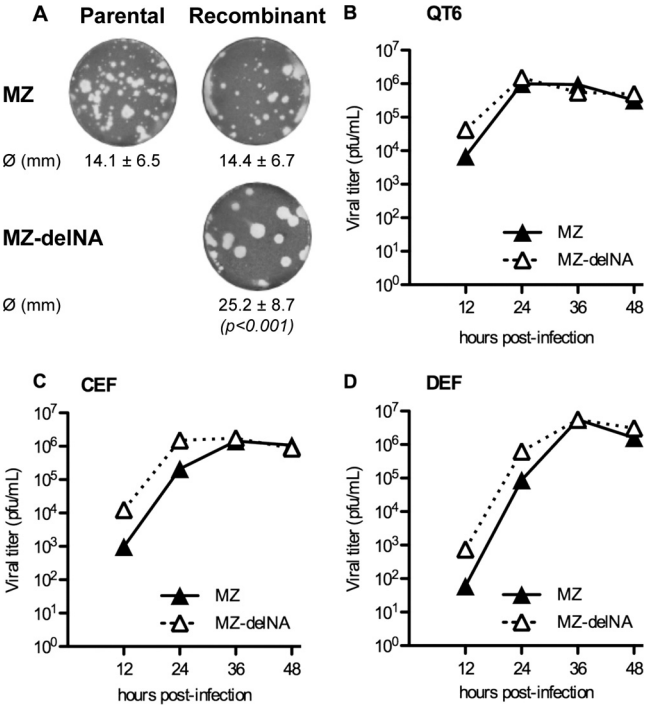

FIG. 2. Growth properties of MZ and MZ-delNA viruses on cultured avian cells. (A) Plaque phenotypes of the parental and recombinant MZ and MZ-delNA viruses assayed on QT6 cells. Cell monolayers were stained with crystal violet after 72 h of incubation at 37°C. Representative data are shown. The mean plaque diameters ± standard deviations as determined for more than 50 plaques are indicated. (B to D) Growth curves of the MZ and MZ-delNA viruses on QT6 (B), CEF (C) and DEF (D) cells. Cell monolayers were infected at an MOI of 0.001 and incubated for 48 h at 37°C. At the indicated time points, the supernatants were harvested and virus titers were determined by plaque assays on QT6 cells. The results are representative of four independent experiments (B and C) or of one experiment (D).

TABLE 2. Enzymatic properties of the MZ and MZ-delNA neuraminidases

Virus	K_m (μM)[a]	V_{max} (fluorescence U/s)[a]	NA/actin ratio[b]	Elution time (h)[c]	
				Without zanamivir	With zanamivir
MZ	23.9 ± 1.3	3.2 ± 0.6	1	1.5	>24
MZ-delNA	22.3 ± 1.5	8.9 ± 1.1	2–3	>24	>24

[a] Results are given as the means ± standard deviations from four independent determinations on duplicate samples of solubilized extracts from 293T transfected cells, using two independent plasmid clones for recombinant NA expression. Similar results were found when whole transfected cell samples were used (data not shown).

[b] The NA/actin ratio was determined by Western blot analysis of the 293T cell extracts used for K_m and V_{max} determination, using an anti-NA antibody and an antiactin antibody.

[c] Elution from chicken red blood cells was performed in the absence or presence of 100 nM zanamivir.

measured at 12 h p.i. were consistently about 10-fold higher for the MZ-delNA virus than for the wt virus (Fig. 2B and C), with the difference being significant only on CEF cells ($P < 0.002$; $n = 4$). At 24 h p.i., a maximum titer of about 10^6 PFU/ml was reached for both viruses on QT6 cells (Fig. 2B). On CEF, MZ-delNA titers were higher than wt MZ titers at 24 h p.i., and a maximum titer of about 10^6 PFU/ml was reached for both viruses at 36 h p.i. (Fig. 2C). The growth kinetics of the MZ and MZ-delNA viruses on DEF, as observed in a single experiment, were similar to those on CEF (Fig. 2D).

Taken together, our data indicate that the MZ-delNA virus has a moderate but reproducible growth advantage in avian cultured cells compared to the MZ virus during the first 24 h p.i.

Enzymatic properties of the neuraminidase of MZ and MZ-delNA. The neuraminidases of the MZ and MZ-delNA viruses were transiently expressed in 293T cells, and their enzymatic parameters were determined on cell extracts using the MUNANA fluorogenic substrate, as previously described (52). The Michaelis-Menten constant (K_m), which reflects the affinity for the substrate, was very similar for the wild-type and the short-stalk NA (23.9 ± 1.3 and 22.3 ± 1.5 μM, respectively) (Table 2) and in the same range as K_m values obtained with other neuraminidases of the N1 subtype (29, 52, 73). The maximal velocity of the reaction (V_{max}), which depends on both the specific activity and the amount of enzyme in the reaction, was 2.8-fold higher for the short-stalk NA than for the wild-type NA ($P < 0.001$) (Table 2). The steady-state levels of the NA proteins in 293T cell extracts were quantified by Western blotting and normalized to actin levels, as described in Materials and Methods. The NA/actin ratio was 2- to 3-fold higher for the delNA compared to the wild-type NA in three independent experiments (Table 2). These data suggest that the higher enzymatic activity detected with the delNA results from a higher level of expression in 293T cells and that the delNA and wt NA proteins have the same specific activity toward the small MUNANA substrate.

Elutions of the MZ and MZ-delNA viruses from chicken red blood cells (CRBCs) were also compared. As shown in Table 2, complete elution of the MZ virus occurred within 1.5 h. In contrast, the MZ-delNA virus was not eluted from CRBCs upon a 24-h incubation at 37°C. In the presence of the neuraminidase inhibitor zanamivir, none of the viruses was eluted, which demonstrated that elution of the MZ virus was resulting from the NA activity. A lower accessibility of the active site of the delNA enzyme toward large substrates is the most likely hypothesis to explain its decreased ability to destroy receptors at the surface of CRBCs, leaving unaltered its ability to cleave the small MUNANA substrate.

Efficiency of production of MZ and MZ-delNA pseudoparticles. In order to investigate whether the deletion in the NA stalk could affect the release of progeny virions from infected cells, we set up an avian cell-based system for the production of viral pseudoparticles (VPP). HIV-1-like particles expressing the MZ hemagglutinin (MZ-HA) alone or in combination with the MZ-NA or MZ-delNA protein were produced from DF1 cells (see Materials and Methods). The efficiency of production of VPP was monitored by measuring the amounts of HIV-1 p24 protein in the cell supernatants by ELISA. The results are shown in Fig. 3. The amounts of p24 antigen in the supernatant of cells coexpressing the MZ-NA or MZ-delNA protein with the MZ-HA protein were increased 1.6- and 1.3-fold, respectively, compared to cells expressing MZ-HA alone (Fig. 3, solid black bars) ($P < 0.05$). This increase was dependent on the viral NA sialidase activity, since it was not observed when the DF1 cells expressed the mutant MZ-NA-Y406F or MZ-delNA-Y406F proteins with no detectable sialidase activity (54) (Fig. 3, solid black bars) or in the presence of 1 μM of zanamivir (Fig. 3, hatched bars). When the DF1 productive cells were treated with a high concentration of exogenous *Clostridium perfringens* neuraminidase (60 mU/ml) in order to control the maximum amounts of VPP possibly released, the p24 antigen content of cell supernatants increased (Fig. 3, solid gray compared to solid black bars). Interestingly, the ratios of the p24 antigen contents measured in the presence and in the

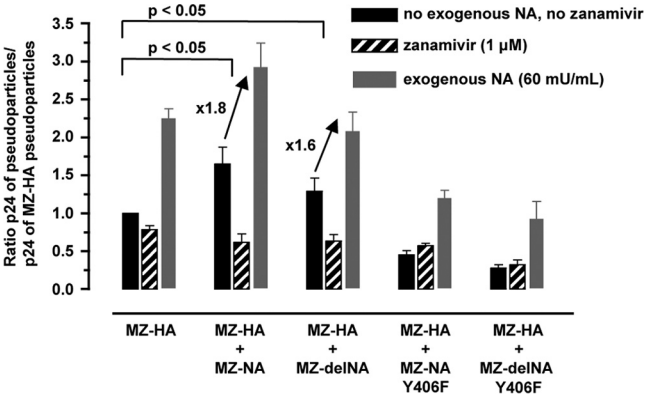

FIG. 3. Efficiency of production of MZ and MZ-delNA pseudoparticles from DF1 cells. DF1 cells were transfected with plasmids allowing the expression of the indicated combinations of proteins. They were incubated for 24 h at 37°C in complete medium, in complete medium supplemented with 1 μM zanamivir, or in complete medium supplemented with 60 mU/ml of exogenous *Clostridium perfringens* neuraminidase. The efficiency of production of VPP was monitored by measuring the amounts of HIV-1 p24 protein in the cell supernatants by ELISA. The results are expressed as the ratio to the amount of p24 protein measured in the supernatants of cells expressing the MZ-HA protein alone in the absence of zanamivir and of exogenous *Clostridium perfringens* neuraminidase and are the means + standard variations from four independent experiments.

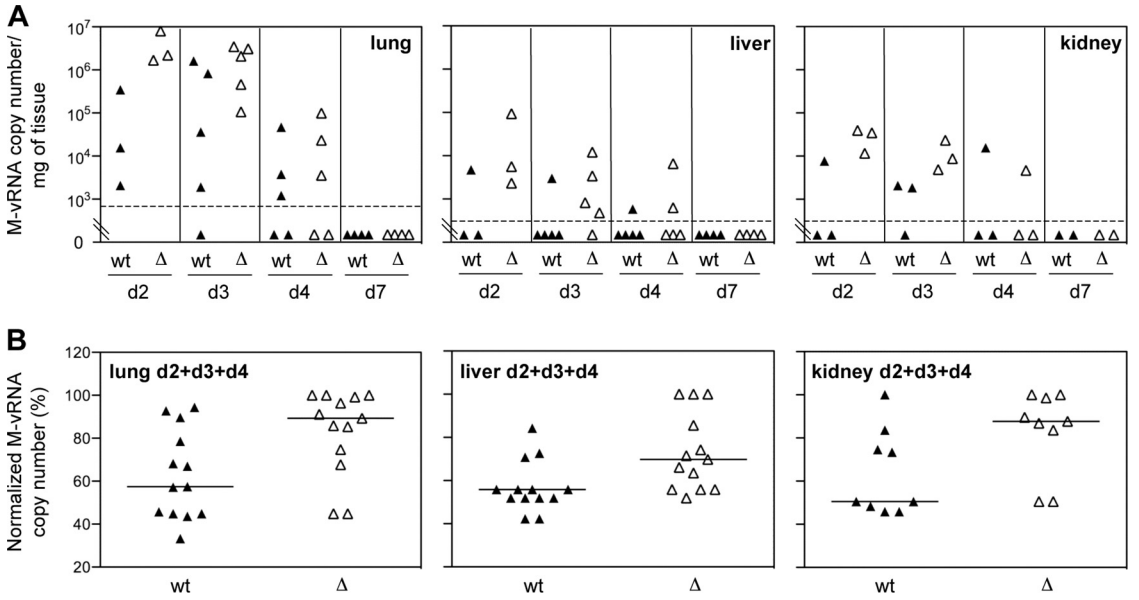

FIG. 4. Levels of M-vRNA in the lungs, livers, and kidneys from MZ and MZ-delNA virus-inoculated chickens. (A) Levels of M-vRNA were determined by qRT-PCR on RNA extracted from the lung, liver, and kidney homogenates of MZ-inoculated chickens (wt, black triangles) or MZ-delNA-inoculated chickens (Δ, white triangles) at days 2, 3, 4, and 7 p.i. The M-vRNA copy numbers/mg of tissue were calculated as indicated in Materials and Methods. The dashed line indicates the positive cutoff values for the lung samples (6.8×10^2 copies/mg) or the liver and kidney samples (3.4×10^2 copies/mg). (B) The M-vRNA copy numbers shown in panel A were converted to their $\log_{10}$ values, expressed as a percentage of the maximum value observed for a given tissue at a given date, and grouped (day 2 plus day 3 plus day 4). The median value for each of the MZ (wt, black triangles) or MZ-delNA (Δ, white triangles) groups is indicated by an horizontal bar.

absence of *Clostridium perfringens* neuraminidase were not significantly different in the supernatants of HA plus NA and HA plus delNA VPP-producing cells (ratios of 1.8 and 1.6, respectively; Mann-Whitney test, $P > 0.05$), suggesting that the deletion in the NA stalk does not increase the efficiency of release of viral particles.

Viral replication in MZ- and MZ-delNA-infected chickens. Four-week-old SPF histocompatible B13/B13 White Leghorn chickens were inoculated intratracheally with 10^7 EID_{50} of MZ or MZ-delNA virus or were mock infected. At days 2, 3, 4, and 7 p.i., tracheal and cloacal swabs as well as blood and tissue samples were taken from euthanized birds for virus isolation, viral RNA quantification, cytokine mRNA quantification, and histopathology and immunohistochemistry analyses.

Viral RNA was extracted from lung, liver, and kidney homogenates, and the levels of M-vRNAs were determined by qRT-PCR as described in Materials and Methods. As shown in Fig. 4A, the highest levels of M-vRNAs were observed at days 2 and 3 p.i. They decreased at day 4 p.i. and were undetectable at day 7 p.i. All lung samples were positive for the presence of M-vRNAs at days 2 and 3 p.i., except for one out of five samples taken from an MZ-inoculated bird at day 3 p.i. At day 4 p.i., only three out of five lung samples were positive in each of the MZ- or MZ-delNA-inoculated groups. The proportions of positive MZ- and MZ-delNA-inoculated birds (cumulative results for days 2, 3, and 4) differed more clearly when liver samples (3/13 and 9/13 positive birds, respectively) and kidney samples (4/9 and 7/9 positive birds, respectively) were examined (Fig. 4A). Moreover, the levels of M-vRNAs in these three tissues, when normalized and grouped (day 2 plus day 3 plus day 4 p.i.) as described in Materials and Methods, were significantly higher in MZ-delNA- than in MZ-inoculated birds ($P < 0.05$, $P < 0.02$, and $P < 0.05$ for lung, liver, and kidney samples, respectively) (Fig. 4B). Very similar results were obtained when the levels of M-vRNAs were determined by qRT-PCR on viral RNA extracted from cecum homogenates (data not shown).

Tracheal swabs and lung and cecum homogenates were inoculated into embryonated eggs for virus recovery. As shown in Table 3, infectious virus was recovered from MZ and MZ-delNA swabs and lung homogenates with similar frequencies, whereas it was recovered more frequently from day 2 and day 3 cecum homogenates of MZ-delNA-inoculated (6/6) compared to MZ-inoculated (1/6) chickens. Most lung homogenates that were negative for virus isolation were also negative for M-vRNA detection by qRT-PCR, except for one and three animals in the MZ-delNA and MZ groups, respectively (Table

TABLE 3. Frequencies of virus recovery from tracheal swabs, lung homogenates, and cecum homogenates from chickens inoculated with MZ or MZ-delNA virus

Virus	No. positive/total on the indicated day p.i.											
	Tracheal swabs				Lung homogenates[a]				Cecum homogenates			
	2	3	4	7	2	3	4	7	2	3	4	7
MZ	2/3	2/3	0/3	1/2	2/3*	3/5*	2/5*	ND[b]	1/3	0/3	0/3	0/2
MZ-delNA	3/3	2/3	1/3	1/2	3/3	5/5	2/5*	ND	3/3	3/3	1/3	0/2
Mock	0/2	0/2	0/2	0/2	ND	ND	ND	ND	ND	ND	ND	ND

[a] Lung homogenates that were negative for virus isolation were also negative for M-vRNA detection by qRT-PCR, except for one animal where indicated by an asterisk.
[b] ND, not determined.

3, asterisks), in agreement with our finding of lower M-vRNA levels in the MZ group. Viral RNAs were extracted from allantoic fluids that tested positive in the hemagglutination assay. The HA and NA segments were amplified by RT-PCR and sequenced. The NA stalk sequence was full length and deleted in the samples derived from MZ- and MZ-delNA-inoculated animals, respectively, as expected. The same sequence heterogeneities in the HA and NA were detected as in the initial viral stocks. They were unlikely to account for the phenotypic differences between the wild-type and MZ-delNA viruses, as similar phenotypic differences were observed in preliminary *in vitro* and *in vivo* experiments using distinct viral stocks which did not show the same sequence heterogeneities (data not shown). Additional sequence heterogeneities or sequence changes were detected in a limited fraction of the samples, possibly resulting from viral multiplication in the chicken or in the embryonated egg (data not shown). Overall, these data strongly suggested that the deletion in the stalk of the NA conferred a replicative advantage to the MZ-delNA virus in chickens.

Tissue lesions associated with viral inoculation. Histological analysis was performed on tissue samples collected at days 2, 3, 4, and 7 p.i. In the lungs, the MZ- and MZ-delNA-inoculated chickens showed lesions typical of a peribronchiolar interstitial pneumonia (Fig. 5B, C, E, and F; Table 4), whereas mock-inoculated chickens showed no significant lesion (Fig. 5A). Signs of interstitial pneumonia consisted of foci of infiltrating lymphocytes and macrophages in the pulmonary parenchyma, distributed mostly at the periphery of bronchiolae. They were more severe in MZ-delNA- than MZ-inoculated chickens at days 2 and 3 p.i. (Fig. 5B, C, E, and F; Table 4) and were of roughly similar intensity at days 4 and 7 p.i. (Table 4). In addition, airway lesions were found in the lower respiratory tract. Necrosis of the respiratory epithelium was noticed as early as day 2 p.i. and was associated with the presence of intraluminal epithelial debris, fibrinous exudate, and rare heterophils at day 3 p.i. These signs of necrotic bronchitis were mild in MZ-inoculated chickens and severe in MZ-delNA-inoculated ones at early times; however, they were more persistent in MZ-inoculated chickens (Table 4). At days 4 and 7 p.i., a major hyperplasia of the respiratory epithelium and a malpighian metaplasia were regularly observed, and these were associated with lumen obstruction in the most severe cases. Finally, the bronchus-associated lymphoid tissue (BALT) was moderatly and severely hyperplasic, respectively, in most MZ- and MZ-delNA-inoculated animals (Fig. 5B and C; and Table 4).

Strikingly, hepatic lesions were noticed in three chickens inoculated with MZ-delNA and euthanized at day 2 p.i. ($n = 1$) or day 7 p.i. ($n = 2$) but in none of the MZ- or mock-inoculated animals. Lesions appeared as large necrotic foci of around 1 mm in diameter associated with inflammatory infiltrates (Fig. 5I) in two animals. Scattered groups of 5 to 10 necrotic hepatocytes, associated with some heterophilic and lymphocytic influx, were observed in the third animal, which was euthanized at day 7 p.i. (data not shown). When terminal deoxynucleotidyl-transferase-mediated dUTP-biotin nick end labeling (TUNEL) was performed on liver sections, a higher level of apoptotic cells was detected on samples from MZ-delNA-inoculated animals from day 2 to day 4 compared to MZ- or mock-inoculated ones ($P < 0.001$) (data not shown). The lymphoid organs of virus-inoculated animals showed some lesions, such as hypertrophy of the splenic ellipsoids, or lymphocytic depletion in the cloacal bursa. The frequency and intensity of these unspecific lesions were similar in the MZ- and MZ-delNA-inoculated groups of animals. No lesions were observed in the other tissues, including the digestive tract, kidney, and brain.

Viral antigen detection in MZ- and MZ-delNA-infected chickens. Immunohistochemistry analysis was performed on lung, liver, digestive tract, pancreas, kidney, and brain tissue samples in order to detect cells positive for the NS1 or NP viral antigens. The NS1 antigen was detected mainly in the cytoplasm and occasionally in the nuclei of scattered cells in the lung parenchyma and in the epithelium of the lower respiratory airways of all virus-inoculated chickens from day 2 to day 7 p.i. but not in mock-inoculated animals (Fig. 5 J to L). The same results were obtained for the NP antigen (data not shown). No difference in signal distribution or intensity was observed between the MZ- and MZ-delNA-inoculated groups of chickens, except for a higher number of positive cells in the lung parenchyma of MZ-delNA-inoculated chickens at day 3 p.i. A few positive cells were recorded in the liver parenchyma at day 2 and from day 2 to day 4 p.i. in MZ- and MZ-delNA-inoculated chickens, respectively (data not shown). No viral antigens could be detected by immunohistochemistry in the digestive tract, pancreas, kidney, and brain samples.

Induction of cytokine and chemokine mRNAs following infection by MZ and MZ-delNA. Cellular RNAs were prepared from the lungs of chickens euthanized at days 3 and 4 p.i. The levels of cytokine and chemokine mRNAs were measured using quantitative RT-PCR and were normalized with respect to the ubiquitin mRNA. The levels of IFN-α, IFN-β, and IFN-γ mRNAs were not significantly different when the MZ-, MZ-delNA-, and mock-inoculated chickens were compared (Fig. 6A to C). The most striking variations were observed for IL-6 and TGF-β4 (analogous to TGF-β1 in mammals) (Fig. 6D and H). Virus-induced transcription of IL-6 mRNAs was observed with both viruses, but the levels of IL-6 mRNAs were about 1,000-fold higher in MZ-delNA- than in MZ-inoculated chickens at day 3 p.i. ($P < 0.02$) (Fig. 6D). The difference between the two groups of virus-inoculated birds was no longer observed at day 4 p.i. (Fig. 6E). The levels of TGF-β4 mRNAs were similar at day 3 but increased at day 4 p.i. in virus-inoculated chickens compared to controls and were about 1,000-fold higher in MZ-delNA- than in MZ-inoculated animals at day 4 p.i. ($P < 0.01$) (Fig. 6G and H).

Smaller variations in response to infection were observed for chemokine mRNAs. Slightly increased levels of mRNAs were measured for CXCLi2 (also called cCAF, analogous to IL-8 in mammals) (Fig. 6F) and for CCL5 (also called RANTES) (Fig. 6I), with significantly higher levels of CCL5 mRNAs being measured in MZ-delNA- compared to MZ-inoculated chickens ($P < 0.01$). The mRNAs encoding CCL4 (belonging to the MIP-1β family) showed the same pattern as CCL5 mRNAs, whereas the levels of CCL20 mRNAs showed no variation upon infection (data not shown).

We questioned whether the differences observed in the cytokine/chemokine response upon inoculation of chickens with the MZ and MZ-delNA viruses could be reproduced upon infection of cultured chicken fibroblasts (DF1 cells) and macrophages (HD11 cells). DF1 and HD11 cells were infected at a high MOI,

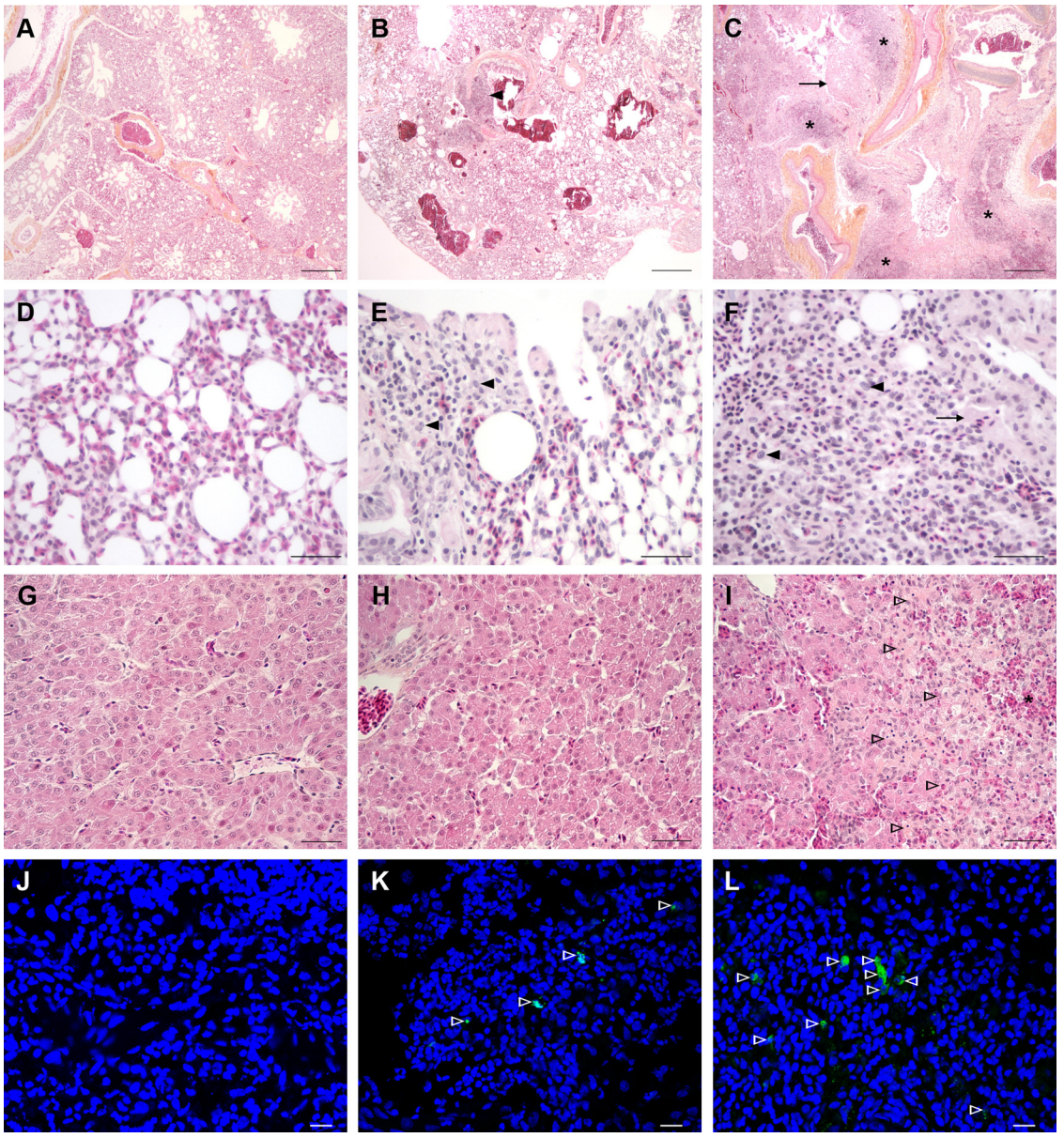

FIG. 5. Histological analysis of tissue sections from MZ and MZ-delNA virus-inoculated chickens. (A to F) Hematoxylin-eosin-safran staining of lung sections at day 2 p.i. Bars, 300 μm (A to C) or 100 μm (D to F). (A) Mock-inoculated chicken. An artifactual presence of some red blood cells in parabronchial lumen was observed. (B) MZ-inoculated chicken. Foci of mild peribronchiolar pneumonia were observed. The bronchus-associated lymphoid tissue (BALT) was hyperplasic (arrowhead). (C) MZ-delNA-inoculated chicken. Signs of severe multifocal coalescing bronchointerstitial pneumonia were visible. The bronchiolae lumen was dilatated and filled with some fibrinous exsudate (arrow) and with debris from the necrotic respiratory epithelium. The lung parenchyma was severely infiltrated by mononuclear cells (*) and some heterophils. (D) Mock-inoculated chicken. Aerian capillaries showed thin walls. (E) MZ-inoculated chicken. Foci of inflammation consisted of mononuclear cells (arrowheads). (F) MZ-delNA-inoculated chicken. Aerian capillaries were occluded by prolific inflammatory infiltrates, which consisted mainly of mononuclear cells (arrowheads) and occasionally contained fibrin (arrow). (G to I) Hematoxylin-eosin-safran staining of liver sections at day 2 p.i. Bars, 100 μm. (G) Mock-inoculated chicken. (H) MZ-inoculated chicken. A normal liver parenchyma was observed. (I) MZ-delNA-inoculated chicken. Foci of hepatic necrosis with trabecular disorganization and pyknotic nuclei (arrowheads), heterophilic infiltration (*), and hemmorhages were observed. (J to L) Immunochemistry analysis of lung sections at day 3 p.i., using an anti-NS1 monoclonal antibody. (J) Mock-inoculated chicken. (K) MZ-inoculated chicken. (L) MZ-delNA-inoculated chicken. The viral NS1 antigen (arrowheads) is detected in the cytoplasm of scattered cells in the lower airway epithelium and in the lung parenchyma.

or mock infected with a control allantoic fluid or with UV-inactivated MZ virus, and were incubated for 6 h at 37°C. Total cellular RNAs were prepared, and the levels of cytokine and chemokine mRNAs were measured as described above. No virus-induced transcription of IFN-α, IFN-β, or IL-6 was observed (data not shown). Compared to those in mock-infected cells, increased levels of mRNAs were measured for TGF-β4, IL-8, and CCL5 in infected DF1 cells and for CCL5 only in infected HD11 cells (data not shown). No significant difference was observed between MZ- and MZ-delNA-infected cells.

TABLE 4. Histological pulmonary lesions in chickens inoculated with MZ or MZ-delNA virus

dpi	Lesion intensity (no. of animals with lesions/total)					
	MZ infection			MZ-delNA infection		
	Interstitial pneumonia	Bronchial lesions	BALT hyperplasia	Interstitial pneumonia	Bronchial lesions	BALT hyperplasia
2	3.0 (2/2)	1.5 (1/2)	3.5 (2/2)	4.0 (2/2)	5.0 (2/2)	5.0 (2/2)
3	3.4 (3/5)	2.2 (3/5)	4.2 (5/5)	4.2 (5/5)	2.8 (4/5)	3.6 (4/5)
4	3.2 (5/5)	3.0 (4/5)	3.8 (4/5)	2.8 (4/5)	4.0 (3/3)[b]	4.7 (3/3)[b]
7	4.0 (2/2)	3.5 (2/2)	1.5 (1/2)	3.5 (2/2)	1.5 (1/2)	4.0 (2/2)

[a] Lesion intensity was scored as follows: 0, none; 1, minimal; 2, mild; 3, moderate; 4, marked; 5, severe. A mean score was calculated for each group of animals.
[b] Only three out of five animals could be evaluated, as the lung sections available for the two remaining animals showed no bronchus.

DISCUSSION

Avian influenza viruses are a main concern for the poultry industry and have also been recognized as a human health concern because of their ability to be episodically transmitted from poultry to humans and to cause pandemics. A better understanding of the molecular mechanisms underlying the adaptation of influenza viruses from the waterfowl reservoir to domestic poultry could contribute to a closer surveillance of their epizootic and zoonotic potential. The data presented here provide evidence that a deletion in the stalk of the NA, a feature that is frequently observed during the process of adaptation of influenza viruses from wild aquatic birds to poultry, favors viral replication and enhances pathogenesis in chickens. The recombinant wild-type MZ virus and its delNA-MZ variant with a 19-amino-acid deletion in the NA stalk, both derived from a low-pathogenicity H1N1 duck isolate, were compared upon intratracheal inoculation in 4-week-old chickens. The variant with a short NA stalk replicated more efficiently and produced more severe pathological lesions than did its wild-type counterpart.

Although intratracheal inoculation of high doses (10^7 EID_{50}) of the MZ or MZ-delNA virus caused no clinical signs, most but not all virus-inoculated birds became infected, as indicated by the successful recovery of infectious virus from lung homogenates prepared at days 2, 3, and 4 p.i. Chickens inoculated with the MZ-delNA virus showed significantly higher levels of M-vRNAs in the lungs at days 2, 3, and 4 p.i. compared to MZ-inoculated chickens, as well as higher num-

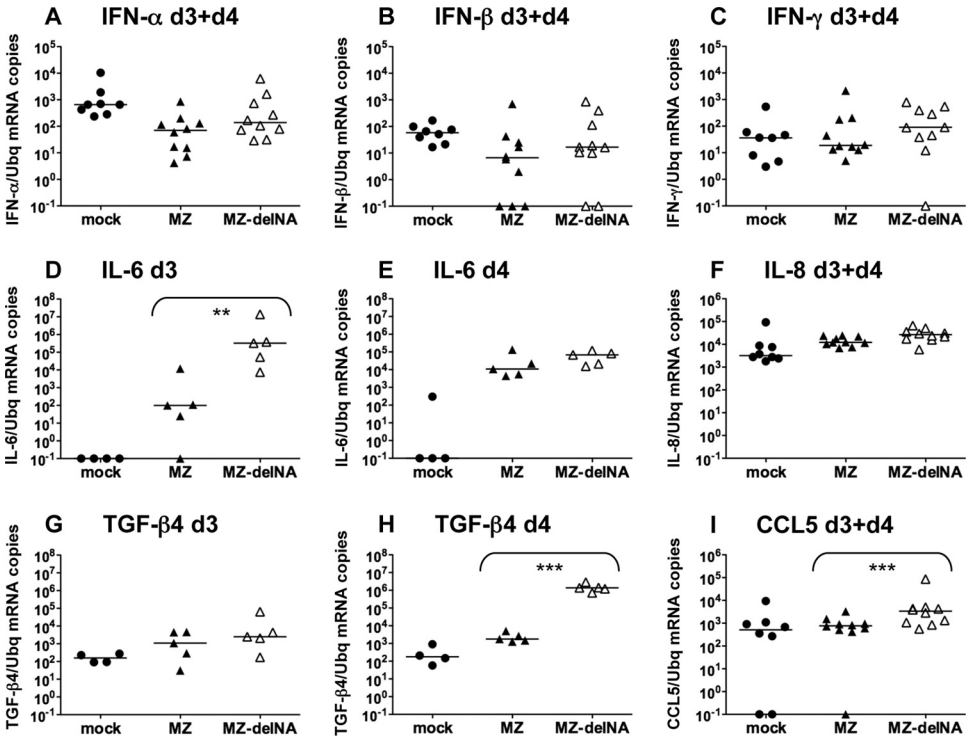

FIG. 6. Levels of cytokine and chemokine mRNAs in the lungs of MZ and MZ-delNA virus-inoculated chickens. Total RNA was extracted from the lungs of five MZ-inoculated chickens (black triangles), five MZ-delNA-inoculated chickens (white triangles), and four mock-inoculated chickens (black circles) at days 3 and 4 p.i. The levels of the indicated cytokine and chemokine mRNAs at the indicated time points were determined using quantitative RT-PCR. The results are expressed as mRNA copy numbers normalized with respect to the ubiquitin mRNA. The median value for each experimental group is indicated by a horizontal bar. Significant differences between the MZ and MZ-delNA groups of animals are indicated by asterisks (**, $P < 0.02$; ***, $P < 0.01$ [Mann-Whitney U test]).

bers of NP- and NS1-positively stained cells in the lung parenchyma at day 3 p.i. Taken together, these observations suggest that the virus with a short NA stalk had an increased replicative efficiency in the lungs. In addition, the MZ-delNA virus showed an increased potential for dissemination to distant organs compared to the wild-type MZ virus. Indeed, animals with detectable M-vRNAs in liver, kidney, and cecum samples were more frequent in the group of MZ-delNA-inoculated birds, and the overall levels of M-vRNAs measured in the liver, kidneys, and cecum were significantly higher for MZ-delNA- than for MZ-inoculated birds. As a strong correlation was found between the levels of M-vRNAs in the lungs, kidneys, and liver, we searched for signs of viral systemic transport. M-vRNAs were detected at a concentration of 2×10^5 to 4×10^6 copies/ml in the sera of all animals that showed infectious virus in the lungs at days 2, 3, and 4 p.i., except for one of the MZ-inoculated chickens (data not shown). However, although infectious virus was recovered from liver, kidney, and cecum homogenates, attempts to recover infectious virus from the serum samples were unsuccessful.

Concomitant with the peak of viral replication observed at day 3 p.i., lesions of insterstitial pneumonia and necrotic bronchitis were observed, which were more severe in chickens inoculated with the MZ-delNA virus. Lesions were observed sporadically in the livers from chickens inoculated with the MZ-delNA but not with the MZ virus; however, no obvious correlation between the presence of lesions and the levels of M-vRNAs could be made. No detectable lesions were observed in the kidneys of MZ- or MZ-delNA-inoculated animals, even when the presence of infectious virus was established. Viral replication in the liver and/or kidney has been observed following natural infection or intratracheal inoculation with highly pathogenic avian influenza virus (30, 45, 75). Like for low-pathogenicity viruses, nephrotropism and nephropathogenicity were observed mostly upon intravenous inoculation in chickens (12, 67, 68). The presence of viral RNA in the kidneys, or renal and liver lesions, was reported following intranasal inoculation of chicken-origin H9N2 or H5N2 virus, respectively (43, 44), but was not observed following intratracheal or intranasal inoculation of waterfowl viruses (44, 67). Our present data demonstrate that the liver as well as kidneys can become sites of active viral replication following intratracheal inoculation of a low-pathogenicity duck-origin influenza virus.

Enhanced viral pathogenesis in the lungs of MZ-delNA- compared to MZ-inoculated chickens was associated with a stronger induction of mRNAs encoding the IL-6 and TGF-β4 cytokines and, to a lesser extent, the IL-8, CCL4, and CCL5 chemokines. These proteins are major mediators of the inflammatory response to influenza infection in mammalian systems (10, 24, 27, 51, 59). The enhanced cytokine and chemokine response in the lungs of MZ-delNA-inoculated chickens most probably resulted from the higher viral loads in these animals compared to MZ-inoculated chickens, since we observed no significant differences between the cytokine and chemokine profiles of cultured chicken cells infected with either the wild-type or delNA virus.

Type I IFNs are thought to play a key role in initiating the antiviral response to influenza infection in mammals (27, 69) and in chickens (8, 35, 64). Low levels of type I IFN mRNAs were observed by us and by others at day 3 p.i. in the lungs of chickens infected with low-pathogenicity viruses (55, 76), whereas a strong induction of IFN-β was reported at earlier times (<36 h) p.i. (1). The highest levels of transcriptional induction and the most significant differences between the MZ-delNA and MZ groups of animals were observed for IL-6 at day 3 p.i. and for TGF-β4 at day 4 p.i. IL-6 is involved in the acute-phase response to influenza infection in mammals and is thought to contribute to the pathological manifestations of the disease in mammals (31, 61, 66). Our results suggest that the avian IL-6 could play a similar role in birds. They are in agreement with data from others who observed a marked increase of IL-6 mRNAs in the tracheae and lungs of chickens inoculated with a low-pathogenicity H9N2 (15, 55) or H11N9 (1) influenza virus. A significant increase of TGF-β activity in mice (7, 16) or in chickens (64) infected with high-pathogenicity influenza viruses was previously reported. To our knowledge, this is the first report of a transcriptional induction of TGF-β in chickens infected with a low-pathogenicity virus. TGF-β, a bipolar factor, acts both as a proinflammatory molecule and as a suppressor of both innate and adaptive immunity (72). It plays a major role in acute lung injury by limiting the inflammation-induced damage and by promoting alveolar repair (60). The higher levels of TGF-β4 mRNAs measured at day 4 p.i. in the lungs of MZ-delNA-inoculated animals may have contributed to the reduced persistence of peribronchiolar pneumonia lesions compared to that in MZ-inoculated animals in our experiments. Additional studies will be needed in the future to address the precise roles and interplay of avian IFN, IL-6, TGF-β, and other cytokines and chemokines in immune cell recruitment and activation, influenza pathogenesis, and viral clearance.

Consistent with the *in vivo* data, a deletion in the stalk of the NA favored replication of the MZ virus on avian cultured cells. Although the sialidase activities of the long- and short-stalk NAs toward a small substrate molecule were the same, the delNA variant did not elute from red blood cells as efficiently as did the wild-type MZ virus. These results are in agreement with previously published data (2, 6, 17, 25, 36, 39) and suggest that the accessibility of the active site of the short-stalk NA to the sialic acids present at the cell surface is markedly reduced. A common hypothesis is that this reduction could counterbalance a decreased affinity of the HA for sialic acids expressed in the respiratory tract of poultry compared to those expressed in waterfowl. Unexpectedly, a growth advantage of the MZ-delNA virus was observed not only on chicken and quail fibroblasts but also on duck fibroblasts. The cultured fibroblasts that were used in our experiments may not accurately reflect the sialic acid contents at the sites of replication *in vivo*. Cultures of differentiated chicken airway and duck intestinal epithelial cells would probably be the most adequate system with which to test this hypothesis.

The NA sialidase activity is essential for the efficient release of newly formed viral particles (11, 57). However, we found that viral pseudoparticles expressing the MZ-HA in combination with the MZ-NA or MZ-delNA protein could be produced from chicken cells with the same efficiency, suggesting that the deletion in the stalk of the NA has little effect on the release of viral particles in this reconstituted system. Alternatively, it could enhance viral entry in chicken cells, as recent studies point to a role of the sialidase activity of the NA at early

steps of the viral cycle (38, 49, 58, 65). Beyond its potential effects on viral replication, the reduced activity of the short-stalk NA could modulate the immune response *in vivo*, e.g., by limiting the stimulation of phagocytosis of virus-infected cells through surface desialylation of macrophages (74) or by regulating the T-cell response through desialylation and activation of the latent TGF-β molecule (48, 59). A potential role of the NA in virus-induced apoptosis has also been proposed (41, 42). Apoptotic cells were more frequently detected in the livers of MZ-delNA- compared to MZ-inoculated animals. However, this difference was most likely related to the higher viral loads in MZ-delNA-inoculated chickens, since we observed no differences in the levels of apoptosis induced in cultured QT6 or HD11 cells upon infection at a high MOI with the MZ-delNA or MZ virus (data not shown).

Influenza viruses with a short-stalk NA have not been isolated from the waterfowl reservoir except in the case of some recent highly pathogenic H5N1 viruses (9, 34), suggesting that such variants either do not have a selective advantage or are being counterselected in aquatic birds. Most human and swine influenza viruses show a long-stalk NA, which indicates that transmission of an avian-origin NA segment to mammals usually occurs in the absence of a deletion in the stalk region. However, by increasing the potential for viral replication in chickens, a deletion in the stalk of the NA is likely to favor the appearance and selection of H5 and H7 viruses with a multibasic cleavage site in the HA and to promote the emergence of viruses with an increased pathogenic potential for humans. The stalk length and sequence were recently shown to affect the virulence in chickens and in mice of reassortant viruses carrying the H5 and N1 genes of a highly pathogenic virus in an A/WSN/33 background (78). Monitoring the NA stalk length should thus be included in the surveillance scheme for influenza viruses circulating in poultry.

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